"Nonhost resistance in *Arabidopsis* (*pen 2-3, pen 3-1, pen1-1, pen 3-1, 2-3*) against *Magnaporthae oryzae*"

Thesis submitted to Department of life science for the partial fulfilment of the M.Sc. Degree in Life Science

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CERTIFICATE

This is certified that the thesis entitled "N**onhost resistance in *Arabidopsis thaliana* (*pen 2-3, pen 3-1, pen1-1, pen 3-1, 2-3*) against *Magnaporthae oryzae*"** which is being submitted by Ms. Gyanaseni Dhar, Roll No-413LS2030, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other institution and university for the award of any degree or diploma.

09/05/2015

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DECLARATION

I do hereby declare that the project report entitled "**Nonhost resistance in *Arabidopsis* (*pen 2-3, pen 3-1, pen1-1, pen 3-1, 2-3*) against *Magnaporthae oryzae***" submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Binod Bihari Sahu, Assistant Professor, Department of life Science, NIT, Rourkela.

Date: 10th May 2015

Place: NIT, Rourkela

GYANASENI DHAR

ACKNOWLEDGEMENT

I am glad to express my sincere thanks to all those people for whom this project has become a successful one.

I wish to express my deep sense of gratitude to my supervisor **Dr. Binod Bihari Sahu** (Assistant Professor, Department of Life Science, NIT Rourkela) for his technical advice, constant support, valuable guidance and encouragement in framing this project.

It is a chance to convey my sense of gratitude to the faculty members **Dr. S.K. Bhutia, (HOD), Dr. Surajit Das, Dr. Bibekananda Mallick, Dr. Bismita Nayak, Dr. Rasu Jaybalan, Dr. Rohan Dhiman,** Department of life science, NIT, Rourkela for their valuable advice and support.

I am also greatly obliged and indebted to Miss Eram Sultan.

A special acknowledgement goes to my friends as well as my lab mates Astha and Abhipsa, for their help and for making the research enjoyable.

My heartfelt thanks to my best friends Bhagyashree Senapati, Shilpa Swagatika Tripathy for their moral support, love and affection.

Last but not the least I would like to thank my parents, my brother, my sister without the blessing of whom I would not have been what I am.

Gyanaseni Dhar

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LIST OF ABBREVIATION

NHR- Nonhost resistance

PRR- Pathogen recognition receptor

MAMPS/PAMPs- Microbial- or Pathogen associated molecular patterns

PTI- PAMPs triggered immunity

ETI- Effectors triggered immunity

HR- Hypersensitive response

MAP-kinase- Mitogen activated protein kinase

ROS- Reactive oxygen Species

PR-Pathogen related

PEN1, PEN2 and PEN3-Penetration gene

ABC -ATP-binding cassette transporter

PDR- Pleiotropic drug resistance

EDS1-Enhanced disease susceptibility 1,

PAD4-phytoalexin-deficient 4

SAG101- senescence-associated gene

SA- salicylic acid

JA -jasmonic acid

NBS- nucleotide-binding site

LRR- leucine-rich repeats

PDA-Potato dextrose agar

ABSTRACT

In India and many countries, most of the people depend on rice, so rice is a staple food of having the economic importance. Many pathogens are present in nature; only for a few pathogens plants are susceptible and resistant. In rice, blast disease is most commonly occurred and a devastating diseases, by which many agricultural loss will be happened. *Magnaporthe oryzae,* a rice fungal pathogen which infect the rice but it resisted by *Arabidopsis* plant. The mechanism of rice resistance to blast disease has been studied elaborately and the combination of "rice *–Magnaporthe oryzae"* patho-system used to describe the plant-microbe interaction easily due to the availability of whole genome sequence and functional genomics approaches. However, "Rice-*Magnaporthe oryzae"* patho system was used as a model. *Arabidopsis* is a model plant for studying NHR against several plant pathogens. Here, nonhost resistance in *Arabidopsis* is studied against rice blast pathogen *M. oryzae*. The infection in *pen 2-3, pen 3-1, 1-1, pen 3-1,2-3* mutants of *Arabidopsis* were higher in comparison with wild type *col-0*. This was evident from confocal microscopy. Furthermore, on studying the expression of *PR1and PDF1.2*, it can be concluded that the pathogen is necrotrophic as *PR1and PDF1.2* expression was present.

Keywords : PTI, PAMPs, PRR,NHR, *pen-2 GFP (wild type) , pen 2-3, pen 3-1, 1-1(double mutant) , pen 3-1, 2-3(double mutant)*

1. INTRODUCTION

Many plant pathogens are present in nature which has distinct phylum and different infection pattern and life cycle; only for a few pathogens plants are susceptible due to the presence of immunity mechanism. Plant exhibited resistance against non adapted pathogen because of an immunity mechanism known as non host resistant mechanism [1, 2]. NHR mechanism were comprise of two types of thought; it may be 1) incompatible activity of non host plants with non adapted pathogen and 2) inability of potential pathogen to cross the immune barrier plant immunity system [3]. Adapted pathogens always want to suppress the plant defence mechanism and change the plant function according to their own need. However, pathogens have an effectors molecule that target distinct plant mechanism [4]. The stability of NHR has been proposed to be composed of several successive layer of defence mechanism that comprise of both constitutive barriers as well as inducible reaction [3,5].Cell wall act as physical barrier and toxic phytoanticipins act as chemical barrier; both are perform as controlling the invasion process of some non adapted pathogen [3,5]. Potential pathogens try to overcome this constitutive defence layer, at that time it need to recognition by plant and induce plant defence reaction. The components which induce NHR and basal resistance are mediated by trans-membrane pattern recognition receptors (PRRs) that recognise slowly evolving microbial- or pathogen associated molecular patterns (MAMPS or PAMPs) [6]. Plant shows the immunity against PAMPs known as PTI (PAMP-triggered immunity) which include MAP-kinase signalling, production of hydrogen peroxide and phenolic compounds included in reactive oxygen species (ROS) at the infection site , ethylene, ion fluxes, transcriptional induction of pathogenesis related (PR) genes, protein phosphorylation and callose deposition at the infection sites and waxy coating barrier on leaves [7]. For pathogenic invasion, plant responses can be classified as two groups 1) PTI and 2) ETI (effector triggered immunity) which is activated by specific strain effectors molecule [6]. Both PTI and ETI help to provide nonhost resistance to plant species against non-adapted pathogen. Although NHR represents the most common and durable form of plant resistance in nature, it is poorly understood at the molecular level [6].

NHR can perform in two layers of defend responses against the biotrophic fungal pathogens (*Blumeria graminis f. sp. hordei*) [8, 10]. The first layer is NHR protect the entry of non adapted pathogen in pre-haustorial stage. Three NHR genes are isolated that are *PENETRATION 1 (PEN1), PEN2 and PEN3*, which required for penetration resistance of

Arabidopsis against the *Blumeria graminis f. sp. Hordei* a non adapted barley biotrophic fungal pathogen. The function of these genes reactived during the prehaustorial stage of pathogen invasion. PEN1encoding for a plasma membrane–anchored syntaxin with a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) domain, which is involved in vesicle fusion and exocytosis of toxic compounds to the pathogen infection sites [9]. PEN2 encodes a myrosinase involved in glucosinolate metabolism in defense responses or a glucosyl hydrolase, which has been located in the peroxisomes [8]. Plasma membrane ATP-binding cassette (ABC) transporter pleiotropic drug resistance (PDR) encoded by PEN3 [10]. These studies demonstrate that *Arabidopsis* NHR to non-adapted biotrophic powdery mildews has two successive and multicomponent defence layers: pre- and post-invasion resistance. Notably, *PEN2 and PEN3* contribute to both stages of resistance [8, 10]. Some studies demonstrated that *PEN2 and PEN3* combinely work to produce and transport toxic chemical towards the infection sites [11]. The first layer of NHR prevents the biotrophic fungal pathogens from forming the haustoria like structure (feeding structure). Those fungal pathogens are overcome the first layer of NHR that encounter a post-haustorial defense mechanism. Some of the genes involved in the second layer of NHR in *Arabidopsis* are enhanced disease susceptibility 1 *(EDS1),* phytoalexin-deficient 4 *(PAD4),* and senescence-associated gene 101 (SAG101) that are factors in post-invasion resistance [8] . Two acids that are salicylic acid (SA) and the jasmonic acid (JA) are play role in defence response and are activated upon infection with biotrophic and necrotrophic pathogens, respectively [12]. SA and JA pathways are involved in the expression of nonhost resistance against the cowpea rust, *Uromycesvignae,* in *Arabidopsis* [13].

In india and many countries, most of the people depend on rice, so rice is a staple food of having the economic importance. Many diseases were caused in rice; one of most serious disease is blast, which is caused by ascomycete fungus *Magnaporthe oryzae*. The mechanism of rice resistance to blast disease has been studied elaborately and the combination of "rice – *Magnaporthe oryzae"* patho-system used to describe the plant-microbe interaction easily due to the availability of whole genome sequence and functional genomics approaches. However, "Rice-*Magnaporthe oryzae"* patho-system is used as a model. Based on host and non-host, the Arabidopsis taken as nonhost and *M. oryzae* as the pathogen, a new model of patho-system is developed.

In this project, I analyse three mutant plants and one wild type. One is single mutant and other two are double mutant. The plants are *pen-2 GFP (wild type), pen 2-3, pen 3-1, 1-1(double mutant), pen 3-1, 2-3(double mutant).* Mutant gene function is opposite to wild type

gene function. *Magnaporthe oryzae* are able to enter and infect the mutant plant. If this experiment is successful, then it prove mutant plants are susceptible and wild type gene are able to provide resistance against pathogen. If this resistance gene are identified and they are able to insert in rice plant, may be *Magnaporthe oryzae* pathogen could not be infect rice plant.

2. REVIEW OF LITERATURE

2.1. Arabidopsis plants choose as a model:

Before the 1980s in plant genetics, the crop and horticulture plants such as maize (zea mays), tomato (*Solanum lycopersicum*), and barley (*Hordeum vulgare*) were used as the model in plant research. Research about these plants in genetic level improved the idea about understanding the plant biology and produces new trait which were provide the benefit for better agricultural breeding. On the time of twentieth century the maize plant played important role in the field of genetic research. One issue with the majority of these early model plant genetic frameworks, including maize, was that their generally expansive size obliged planting outside by utilizing farm machinery, huge numbers of scientists in urban regions were not available and also they have the long generation time for which they could not be studied continuously in a year.

Scientist Friedrich Laibach in 1943 was first promoted *Arabidopsis* as a model organism in plant research. *Arabidopsis* plant have many attributes for being as a model, such as 1) It has smallest genomes in the plant kingdom: 135×10^6 base pairs of DNA distributed in 5 chromosomes ($2n = 10$) and almost all of which encodes its 27,407 genes 2) The life cycle is short-about 6 weeks from germination to seed maturation 3) Seed production is prolific and the plant is easily cultivated in restricted space 4)Transformation is successful by utilizing *Agrobacterium tumefaciens* 5). Mutations can be easily generated (e.g., by irradiating the seeds or treating them with mutagenic chemicals) 6) It is normally self-pollinated so recessive mutations quickly become homozygous and thus expressed 7) *A. thaliana* is studied by a multinational research community in industry, government and academics.

Maarten Koornneef and co-workers at Wageningen Agricultural University identified a large number of genes by the process of mutation and mapped them to chromosomes, establishing the genetic map of the *Arabidopsis* genome. However, discovering the genetic analysis by using mutant was demonstrated by Christopher Somerville, working at the University of Illinois and Michigan State University. Christopher Somerville was demonstrated to characterize the important biochemical processes such as photorespiration, lipid biosynthesis by studies the genetic analysis. Elliot Meyerowitz (California Institute of Technology) and co-workers determined that the amount of DNA in the Arabidopsis genome was smaller than any other seed plant.

Classification of Arabidopsis thaliana:

Kingdom-Plantae (plant)

Subkingdom-Tracheobionta (vascular plant)

Superdivision- Spermatophyta(Seed plants)

Division-Magnoliophyta(flowering plants)

Class-Magnaliopsida(Dicotyledons)

Subclass-Dilleniidae

Order-Capparales

Family-Brassicaceae (mustard family)

Genus-*Arabidopsis Heynh* (rock cress)

Species: *Arabidopsis thaliana* (L.) Heynh. (mouseear cress)

Figure-1(classification and picture of *Arabidopsis* plant)

Mutant genes are formed from wild type by recessive mutation and they performed their function in opposite manner to wild type gene. Double mutant are more prone to infection than single mutant. Many penetration genes are present and they encode different protein and performed differently in function. Their mutant are formed that are *pen 2-3* (3 shows the changes in 3 position on pen 2 gene), *pen 3-1, 1-1* (double mutation that is mutation in pen 3 and pen 1), *pen 3-1, 2-3* (double mutation that is mutation in *pen 3* and *pen 2*). These mutant

genes are prone to infection and show susceptible towards pathogen. There are some mechanisms to describe the immune system of plant.

The following genes are arranged clearly on the chromosome of *Arabidopsis*, such are *PEN 1, PEN2, PEN 3, AGT 1, NPR 1, PAD 4, RAR1, PMR2, PMR 2, MPK6, SSI2.*

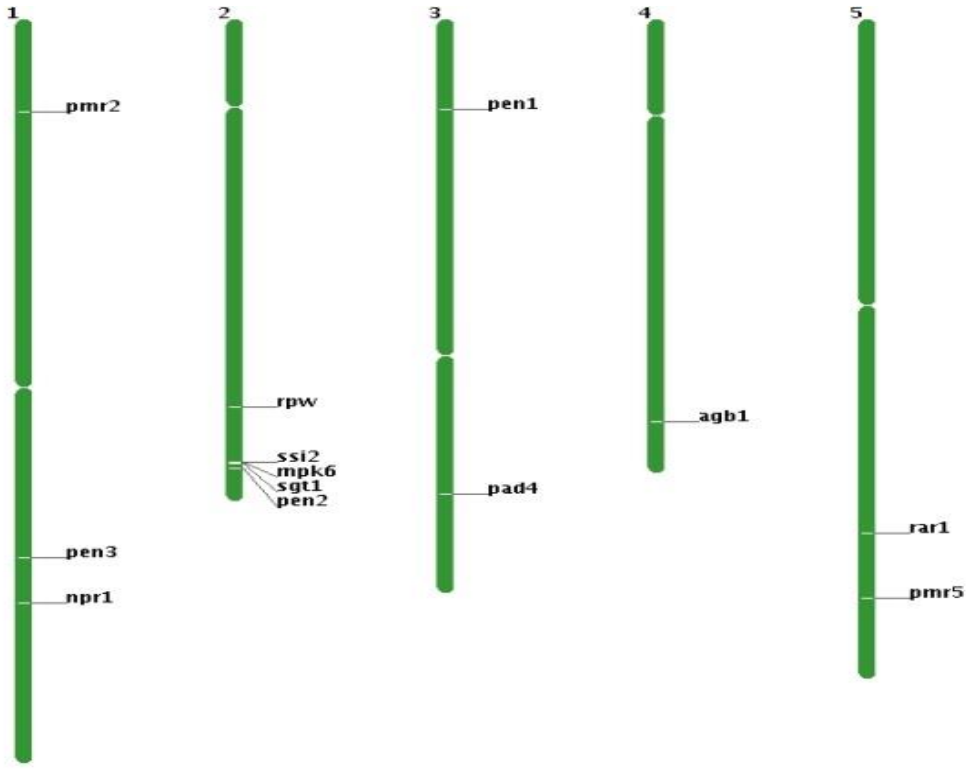


Figure-2 Location of nonhost resistance genes across the genome of *A. thaliana*.

2.2. Pathogen sensing and host defence:

Plants do not have adaptive immune system as that of animal in respond to pathogen. Plants are successfully detecting the pathogen and show their defence effect against pathogen that occurs by some genes which encoded in the genome. Recognition and in respond to pathogen challenge, plant contains two distinct defence mechanism [15]. Bacterial flagellin, lipopolysaccharides and fungal-oomycete cellulose-binding elicitor proteins, these are referred as PAMPs (Pathogen-associated molecular patterns) ,which are recognized by transmembrane receptor called as PRR (PAMPs recognition receptor) in plant that initiate the basal defence activation, which demonstrates a first line of defence against pathogen that is

reminiscent of innate immunity in vertebrates [16,17]. Plant reflects their defence responses against pathogen referred as PAMP-triggered immunity (PTI) [18]. A hypothetical view show in both plants and animals that a biological 'arms race' is occurring ,in which pathogen have able to acquired mechanism to which they cross the defence barrier of PAMP-triggered immunity by evolving effectors molecules. Effectors molecules have the capacity to change the defence system of the host cell, by which disruption of first line of defence could be occurred. Plant evolution has evolved with some proteins that detect specific effectors molecules by a mechanism called as 'effectors-triggered immunity' [15] that represent a second line of defence. The genes that encode the effectors-triggered immunity are known as resistance (*R*) genes. Most *R* genes encode proteins that composed of a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs). NBS-LRR proteins are involved in the recognition of specialized pathogen effectors (also called avirulence (Avr) proteins) that are thought to provide virulence function in the absence of the cognate *R* gene [15]. Accordingly the sequence that identifies the NBS domain, the NBS-LRR protein can be divided into two classes that are TIR and non-TIR. The TIR class of plant NBS-LRR proteins contain an amino-terminal domain with homology to the Toll and interleukin 1 receptors. The non-TIR class of NBS-LRR proteins contain α-helical coiled-coil–like sequences in their amino-terminal domain [19].

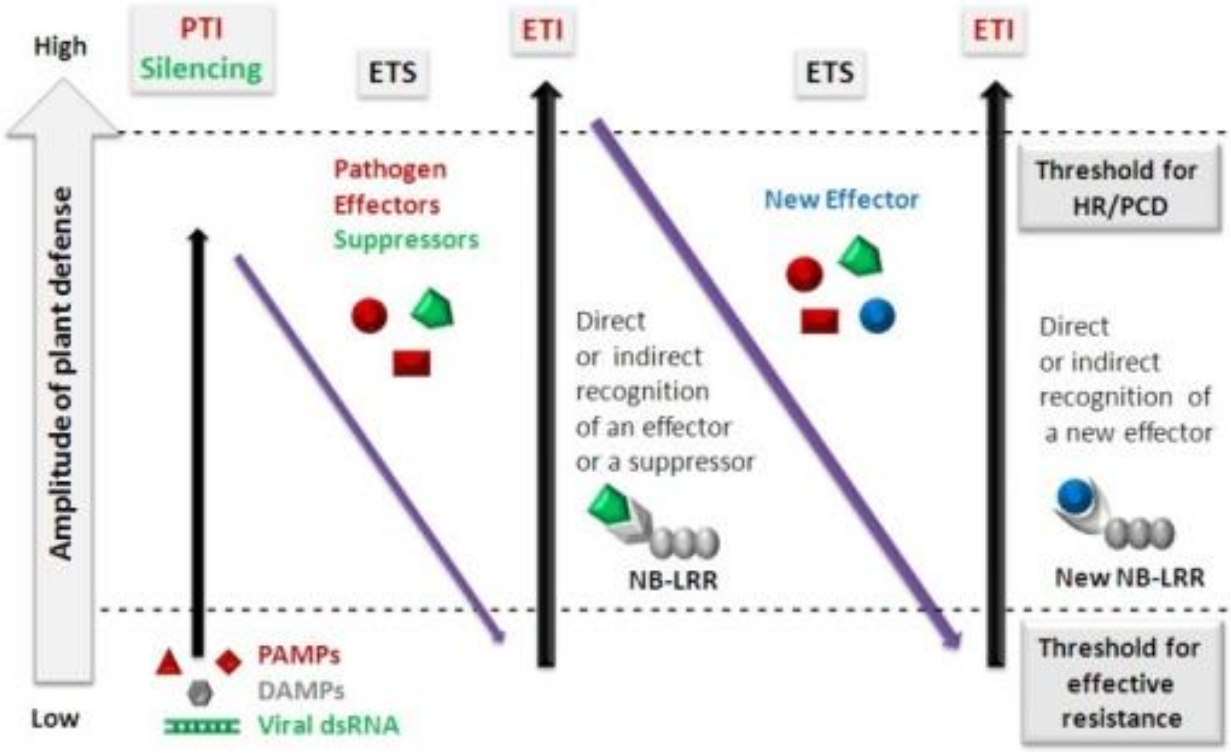


Figure-3 ZIG-ZAG model for nonhost mechanism

(taken from Jones and Dangl nature, 2006)

NBS-LRR proteins and pathogen effectors interaction are two types

1) Direct interaction 2) indirect interaction.

The first evidence for direct interaction studies proved by studied of Pi-ta and R gene from rice that show resistance to specific strain of the rice blast fungus *Magnaporthe grisea,* which evolve the effectors AVR-Pita [20]. Interaction of the functional portion of AVR-Pita with the LRR like domain of Pi-ta could be detected by Yeast two-hybrid experiments. Another model was examined which was support the direct detection method ,that the observation in *Arabidopsis thaliana* RRS1 protein interacts with the bacterial wilt pathogen protein PopP2 in a 'split-ubiquitin', which was proved by yeast two hybrid experiment [21]. RRS1 is an atypical member of the TIR-NBS-LRR class of resistance proteins because it contains a carboxy-terminal WRKY domain [22]. Notably, the inactive form of RRS1, RRS1-S, can also bind to PopP2 in that assay, suggesting that either the interaction in yeast does not recapitulate the interaction in plants or that steps in addition to legends binding are necessary for the activation of signalling.

The example of an indirect recognition mechanism in the *Arabidopsis thaliana* is, Arabidopsis proteins RPS5and PBS1 detected the *P. syringae* effector AvrPphB. RPS5 is a plant NBS-LRR,whereas PBS1 is a protein kinase with unknown substrates [23–24]. Both proteins are requiredfor the recognition of AvrPphB in *P. syringae* strains. Direct interaction between RPS5 and AvrPphB has not been detected; however, the interaction between both AvrPphB and RPS5 with PBS1 and resulting a ternary complex (J. Ade and R.W.I). AvrPphB is a cysteine protease which cleaves PBS1 at a specific site [25, 26]. Therefore, it seems that RPS5 functions to detect pathogen effectors such as AvrPphB by monitoring the status of PBS1.

AvrRpm1 and AvrB are two effectors protein isolate from the *Pseudomonas syringae* a bacterial pathogen, both are recognized by RPM1 of NBS-LRR protein in *A. thaliana,* whereas another type of effectors protein from *P. syringae* is AvrRpt2 (cysteine protease type III effectors), which is recognized by *A. thaliana* NBS-LRR protein RPS2 [27, 28]. The direct interaction between effectors molecule and *A. thaliana* NBS-LRR protein has not been detected. However, RIN4, another plant protein is linked with AvrB, AvrRpm1 and AvrRpt2 [29, 30]. RIN4 bind to both RPS2 and RPM1 that giving the indirect recognition patterns of AvrRpt2, AvrB andAvrRpm1. The binding patterns provide functional changes in the RIN4

protein for which RIN4 phosphorylated and proteolytically cleaves by AvrRpt2 [29, 30]. Therefore, the Arabidopsis basal defence responses is negatively regulated by RIN4 protein.

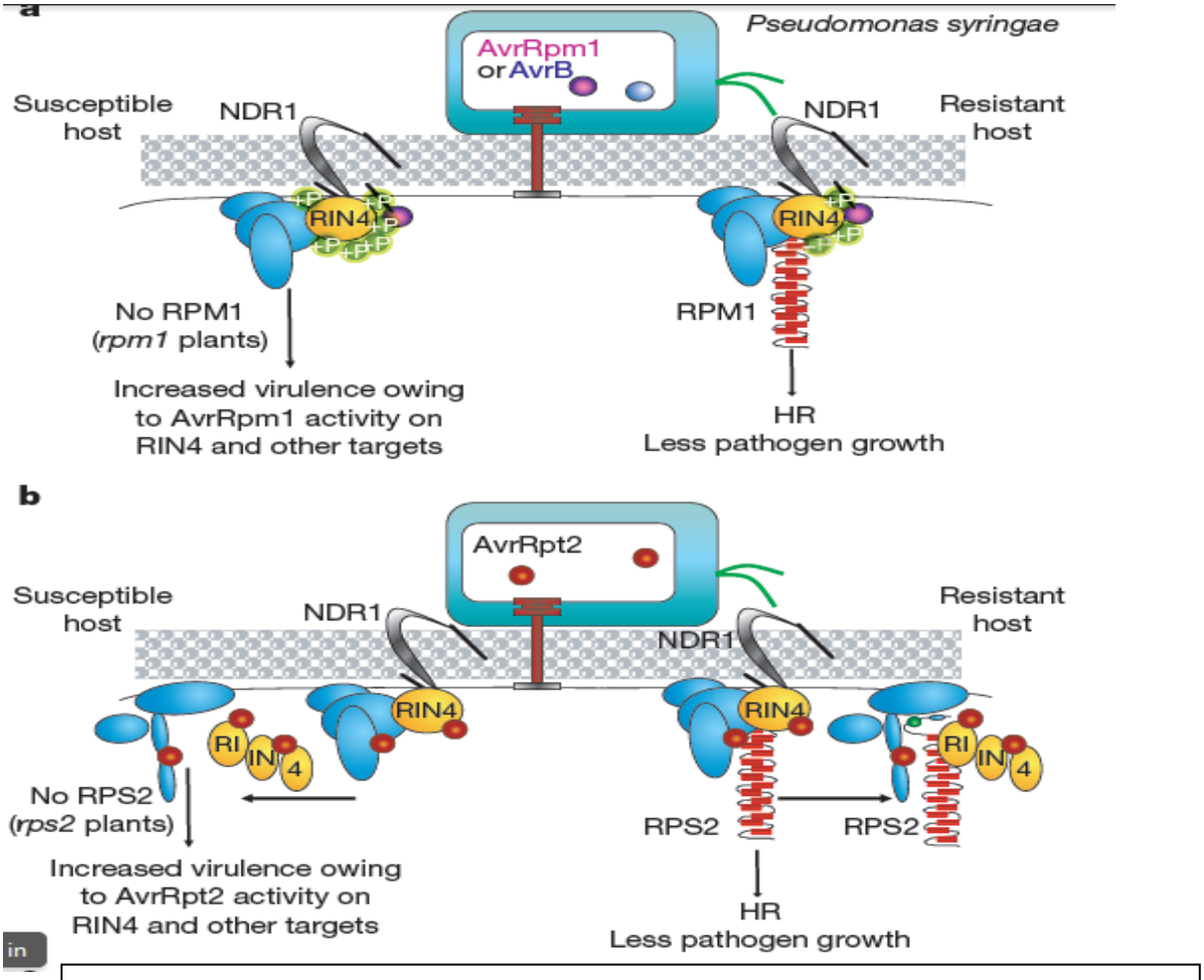


Figure-4 Plant immune system activation by pathogen effectors that generate modified self molecular patterns.
(picture taken from Vol 444j16 November 2006jdoi:10.1038/nature05286)

The location of LRR domains in NBS-LRR protein at the site of carboxy terminal. Crystallization of non-plant protein LRR domains have been occurred, as a result a barrel like structure aligned with parallel β-sheet lining is formed, which is situated at the inner concave surface and the rest of space is occupied by α-helical structure. LRR domain is involved in detection pathogen effectors molecule and helps to support several evidence in NHR hypothesis. However, it is thought that protein-protein interaction in animal system is mediated by LRR domain. Pathogen effectors targeted a protein in plant which is present with NBS-LRR protein. The amino-terminal domain of the NBSLRR protein mediated the interaction of pathogen target–NBS-LRR. As a result, a tightly folded complex structure is formed which is consist of amino terminal domain, NBS, LRR and effectors target part.

Adenine nucleotide is bound with NBS domain that confirms the conformation of NBS domain. In particularly the interaction shows negative regulatory function.

Effectors induce the conformational changes in host protein and help to exchange of ADP for ATP. That brings the change in nucleotide bounded with NBS domain, which in turn again changes the NBS-LRR domain structural arrangement. Activation in NBS-LRR protein represented the structural changes and binding status of nucleotide. These alterations inducing a new binding site for downstream signalling molecules and signalling pathways activation was resulted.

Arabidopsis thaliana represents as a host for *Erysiphe cichoracearum*, a powdery mildew but it is represent as nonhost for *Blumeria graminis-f. sp. hordei (Bgh*),a the barley powdery mildew. Successful penetration and rapid proliferation of the fungus on *Arabidopsis* were resulted from the interaction between Arabidopsis and *E. cichoracearum.* When Bgh attack on Arabidopsis, Bgh first germinate at the surface but sometimes succeeds to enter the cell. As a consequence, the nonhost/pathogen *Arabidopsis*/ *Bgh* interaction provides an excellent model system for studies on penetration resistance and the contrast between the host and nonhost interactions can be used to compare successful vs. failed penetration events.

Cell wall appositions, known as papillae formed in the plant. Papillae have contain callose, lignin, phenolic compounds, reactive oxygen species, and proteins and are act as a physical barrier to opposes penetration which is cause by the fungal pegs penetration [31;32]. however, By analysis a number of molecular component by using forward genetic screens that show the Arabidopsis ability to create a potential for resist the non-adapted powdery mildew fungus *Blumeria graminis f. sp. Hordei* (barly pathogen)[9,10]. Arabidopsis populations were mutagenized with Ethyl methane sulphonate (EMS) which were screened for mutants that enhances to increasing Bgh penetration frequencies. Penetration which is successful by Bgh that resulted to vidualize whole cell in autofluorescence [9] and encasement of the nascent haustorium in callose, which could be analyze by microscopically after inoculated tissue staining with the aniline blue(fluorescent dye) [10]. Further studied about mutant screen, several penetration (pen) mutants enhance the higher frequency in Bgh entry into epidermal cell. Three of these mutants, designated as pen1, pen2, and pen3, have been characterized and the corresponding genes identified. The *pen1* allows to increased penetration phenotype which suggests that the nonhost fungus encounters effective barriers to penetration that are defective in the mutant. There are at least three potential barriers to fungal penetration. Although this is poorly documented, the first two barriers to fungal penetration are to be cuticle and the primary cell wall [33]. Papillae which are formed after

infection are representing a third barrier to fungal infection. As a plasma membrane syntaxin helped in secretion, PEN1 could be involved in the deposition of cuticle precursors, of the primary cell wall and of papillae. Its role in these processes remains to be determined.

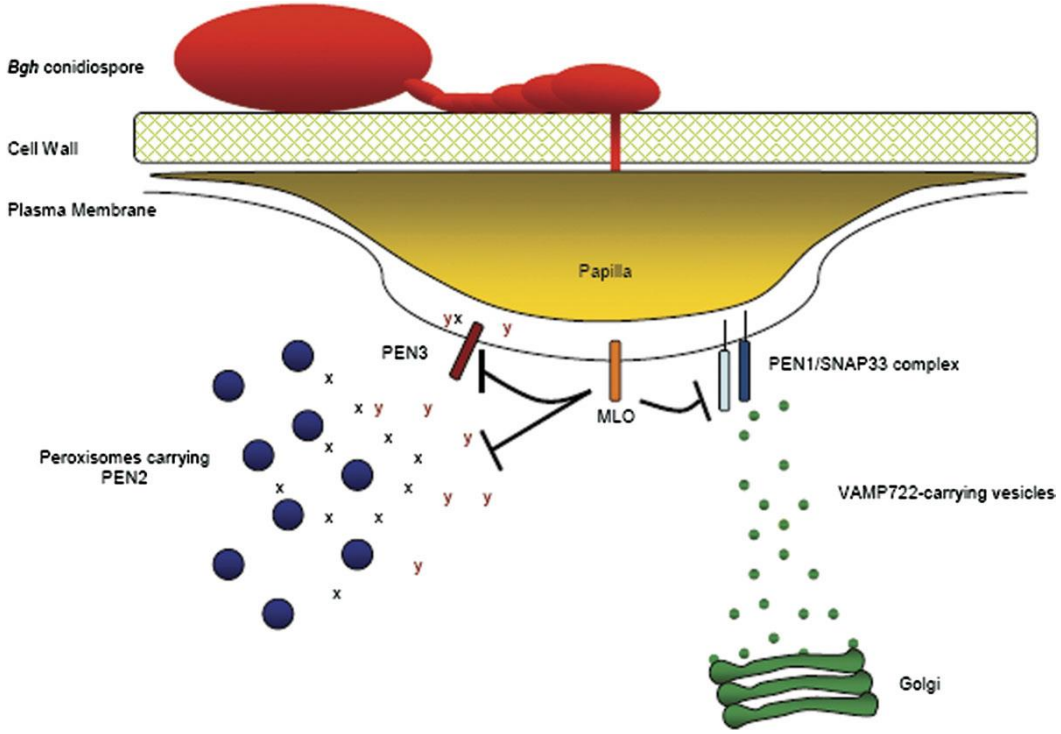


Figure-5 Model depicting the roles of the PEN proteins and MLO in resistance to penetration by powdery mildew fungi. X and Y represent putative PEN3 transport substrates

An example of host and pathogen interaction is Powdery mildew *E. cichoracearum* infection in Arabidopsis is studies extensively [34]. On the studies about the interaction, within 24 h after inoculation (hai), powdery mildew germinate conidia that developed into appressoria, a penetration peg was developed from appressorium which was help in penetration the outer epidermal cuticle and cell wall in host cell, that developed to established a haustorial complexes within epidermal cells. Further development permitted the pathogen to take water, nutrient and mineral from host plant and allows further hyphal growth on surface of the leaf.

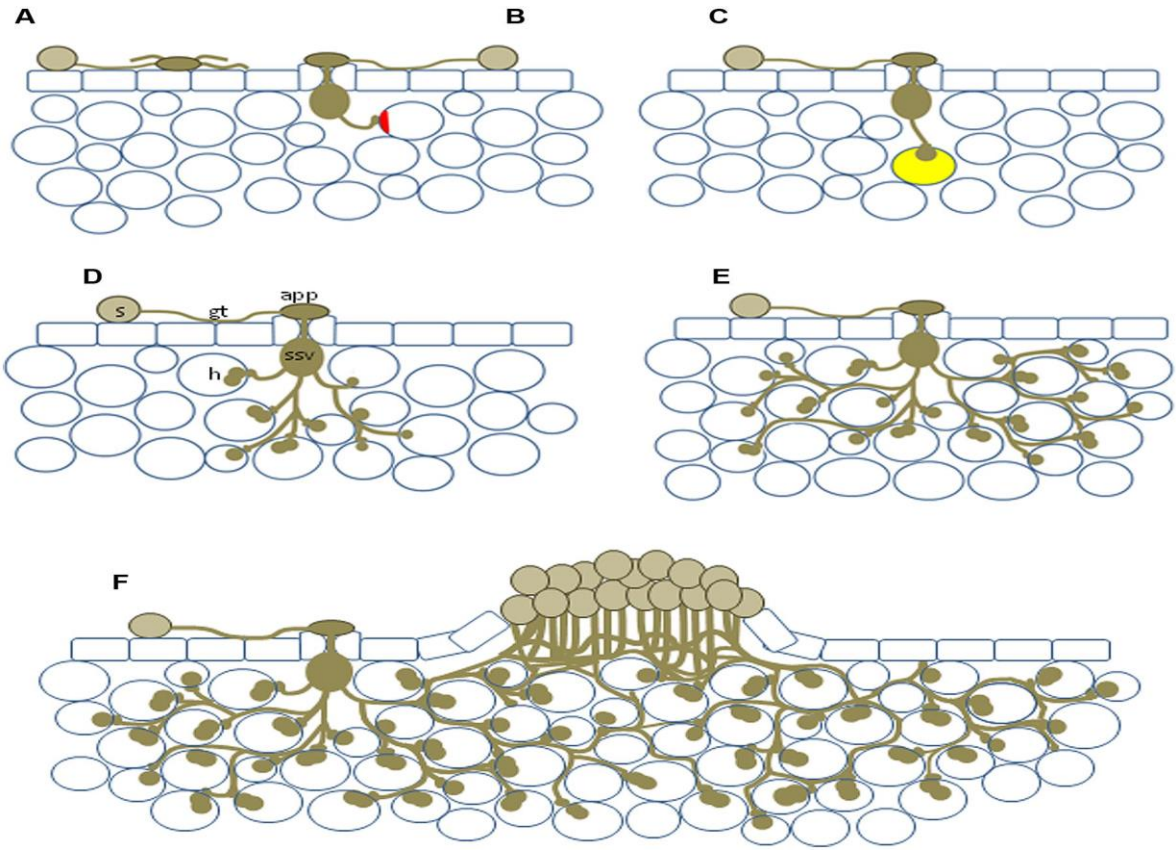


Figure-6 Diagram showing the range of potential NHR outcomes. (A) A spore germinates to produce an aberrant appressorium-like structure (B) pre-haustorial resistance in which a germination event enters the leaf but is unable to penetrate the cell wall, plant cell wall appositions (shown in red) can occur(C) infection resulting in the formation of a single haustorium, autofluorescence (yellow) can be associated with these events;(D) haustoria produced in multiple plant cells; (E) relatively large infection site encompassing numerous mesophyll cells but sporulation never observed; (F)formation of a sporulating uredinia.

TABLE:1 List of host and nonhost plants studied by people

Host	Non host	pathogen
Arabidopsis thaliana		*Erysiphe cichoracearum*
	Arabidopsis thaliana	*Blumeria graminis f. sp Hordei*
Oryza sativa		*Magneporthae oryza*
	Arabidopsis thaliana	*Magneporthae oryza*
Arabidopsis thaliana		*Botrytis cinerea*

2.3. *Magnaporthe oryzae* Pathogen

Pathogen in broad sense defined as a disease causing agent. Pathogens are different types that are viruses, bacteria, fungal. *Magnaporthe oryzae* is a fungal pathogen which is responsible for causing disease in rice called as rice blast. The rice–M. Oryzae pathosystem has consider as a best model for plant–microbe interactions study. *M. oryzae* infect the rice by following many developmental processes. In the first developmental process, a germ tube produced from conidium and grows towards a infectious structure called appressorium. Appressorium secrete a substance like mucilage by which it tightly adheres to the surface of the plant. Fungus produces turgor pressure under the appressorium which is melanin lined, due to this turgor pressure a narrow penetration peg is produced towards the host surface. Fungus is able to enter into a leaf epidermal cell through penetration peg. After entry, the peg give rise to bulbous and lobed infectious hyphae which grow intra- and intercellularly.

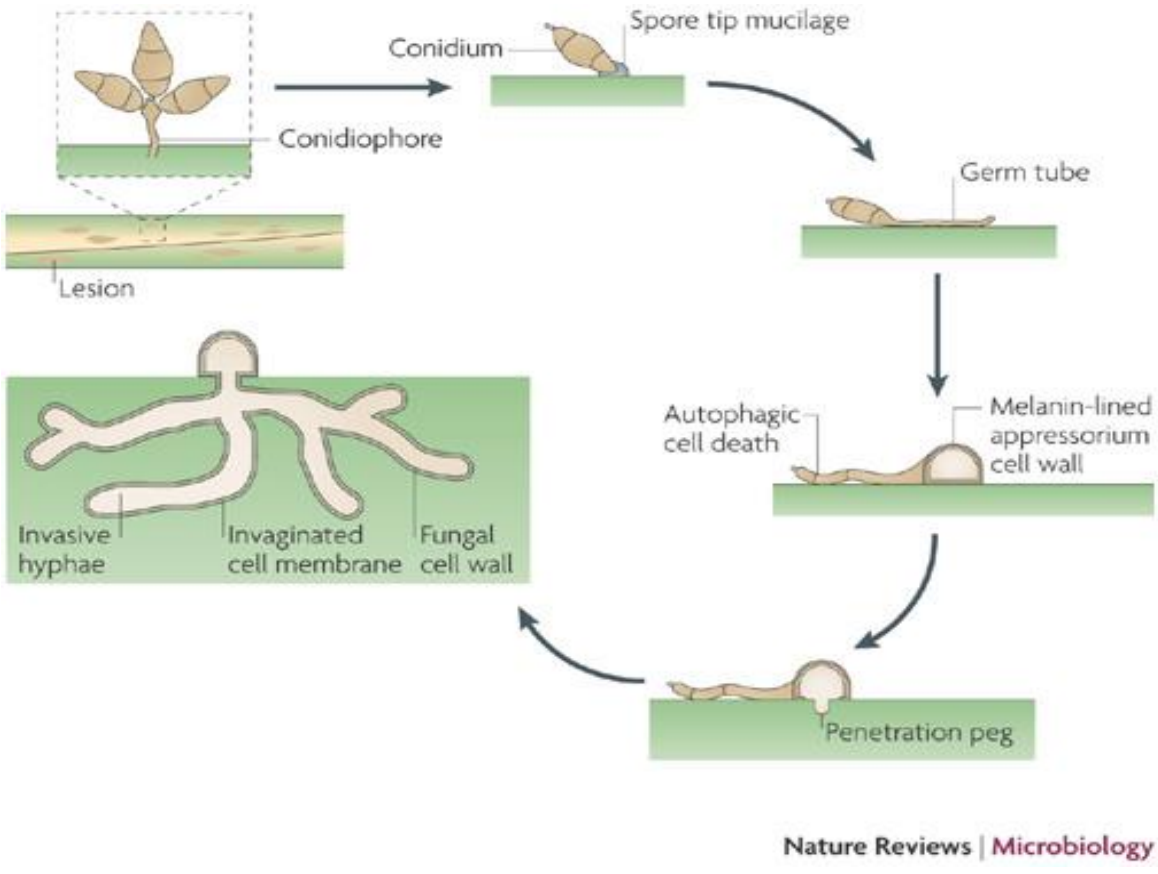


Figure-7 Life cycle of *Magnaporthe oryzae*

Arabidopsis act as nonhost for *M. oryzae* but the mutant species with specific genes are act as host for *M. oryzae*. *PMR5* and *AGB1* gene in *Arabidopsis* give NHR to *Arabidopsis*

against *M. oryzae*. *PMR5* and *AGB1* gene give penetration resistance to *Arabidopsis* which indicate a genetic network regulated the resistance [35]. On the study about genetic network, *pen2 NahG pmr5, pen2 NahG agb1, pen2 pmr5 agb1,* and *pen2 NahG pmr5 agb1* mutants were generated. *M. oryzae* infects the mutant but it shows that the penetration rate is higher in double mutant than single mutant. *M. oryzae* penetrated through the epidermal cell but further it could not grow intra or intercellular in *Arabidopsis pen2 NahG, pen2 pmr5,* and *pen2 agb1* mutants due to *PMR5* and *AGB1*.

3. OBJECTIVE

- To study the phenotype of leaves of the *Arabidopsis* mutant after infection.
- To visualize the infection by trypan and aniline blue staining.
- To check the DNA integrity after infection by *M. oryzae*.
- To study the involvement of defense pathway genes during infection.

4. MATERIALS & METHODS:

4.1.SAMPLE PREPARATION

4.1. a. Plant material:

Arabidopsis seeds were collected from "Nottingham Arabidopsis Stock center" (NASC) and stored at 4°C. The *Arabidopsis* accession code was Col-0. I used the mutant *pen 2-3, pen 3-1, 1-1* (double mutant), *pen 3-1, 2-3* (double mutant). Then, seed samples were soaked in distilled water in a 1.5 mL Eppendorf tube overnight (that is needed for good germination and breaking the dormancy).

4.1. b Soil preparation:

Agro peat soil was mixed with vermiculite in the ratio 1:5, mixed evenly. Then pots were filled with the mixture of soil.

4.1. c. Fertilizer preparation:

TABLE-2

Name of chemical	amount
Ammonium nitrate	6.516g in 1000ml
Potassium chloride	7.6249g in 1000ml
Disodium hydrogen phosphate	1.824g in 1000 ml

100mL was taken from each stock solution and added water to maintained volume 600mL and pouring each tray.

4.1.d. Plant growth

Seeds were then sowed on mixture of soil then covered it with plastic film so that humidity could be maintained. Light and temperature were maintained. Light should be maintained and temperature of plant growth chamber was maintained at 21°C. After 3 days, uncovered the tray and seedlings formation occurred. For the better growth of plant water and

fertilizer were given to the plant alternately. After 11 days plantlet were transplanted individually in the pot. For maintaining the humidity the tray was covered for three days.

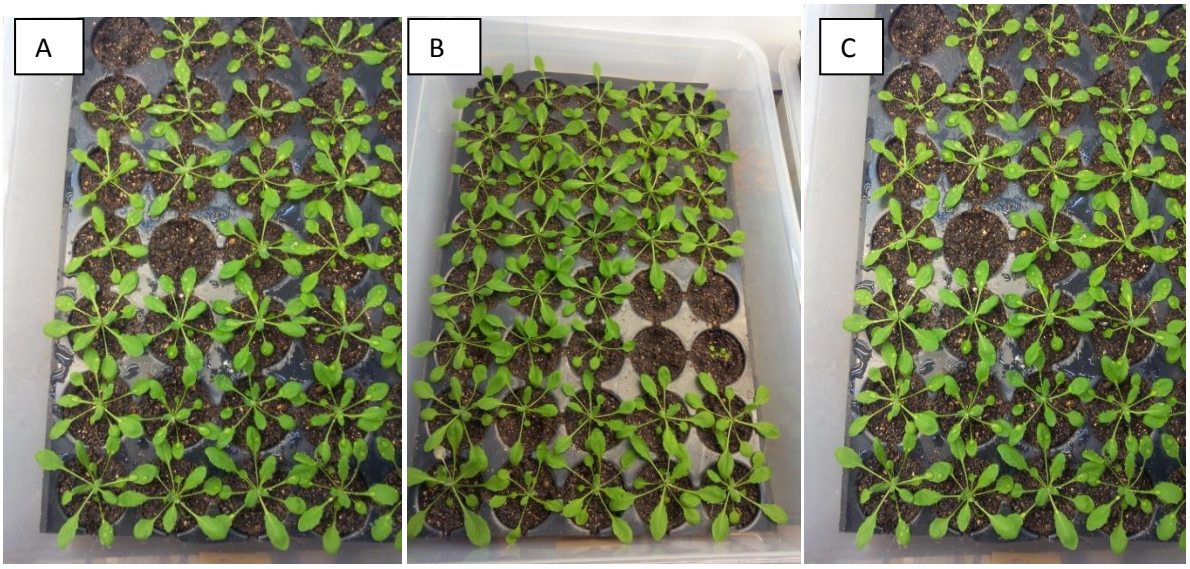


Figure-8 Growth of mutant type Arabidopsis plant A) p*en 2-3* B) *pen 3-1, 1-1* C) *pen 3-1, 2-3*

4.2 FUNGAL MATERIAL

Fungus *Magnaporthe oryzae* was collected from National Center for Plant Genomic Research (NCPGR) of strain Himalayan isolate. *Magnaporthe oryzae* isolate was incubated on oatmeal agar media and potato dextrose agar (PDA) media (with Agar @1.5%) in petridish at 25°C. Then, the inoculum was prepared by washing the petriplates having the mycelia of 7d old growth by distilled water. In order to inoculate *M. oryzae*, spores were diluted 10 µl droplets (10^5spores/ml). In the culture plates water was added, shake the plate, then transfer the spores in the falcon tube.

4.2. a. Oat meal agar media: (for 100mL)

For 100ml media, 6.00gm of oat meal powder (HIMEDIA) and 1.25 gm of agar (HIMEDIA) was dissolved in 100mL of distilled water and pH of 7.2±0.2 was maintained. Then autoclaved at 121°C at 15 lbs pressure for 15-20 minutes for sterilization. Then 100 µl of streptomycin was added in it before pouring in petridish.

4.2. b. Potato dextrose agar media :(for 100mL)

In 100ml PDA media, 1.3 gm of potato dextrose agar (HIMEDIA) and 1 gm of agar (HIMEDIA) was mixed in 100 mL of distilled water. Then autoclaved at 121°C at 15 lbs pressure for 15-20 minutes for sterilization. Then 100 µl of streptomycin was added in it before pouring in petridish. Then about 25µl of media was poured in each petridish.

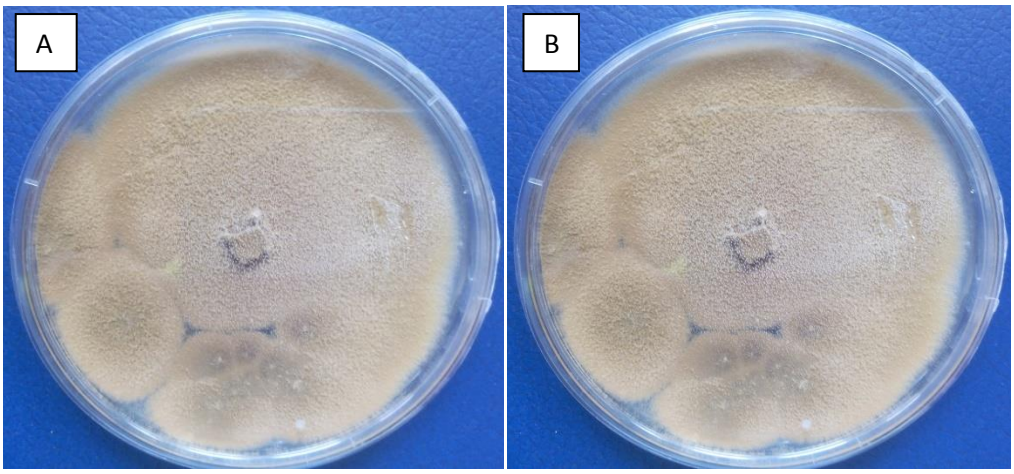


Figure-9 Growth of *Magnaporthe oryzae* A) Growth on Oat meal agar media B) Growth on Potato dextrose agar

4.3 LEAF INFECTION

For fungal infection, autoclavable petriplate (150mm* 25mm, HIMEDIA) was taken; Whatman paper was placed on the petriplate. Plant leave of 3-5 weeks old plant was cut and arranged in a triplet. In the first triplet on the moistened paper, water was placed on the surface of the leaf. Then, 10µL of spores was placed on all other triplet leaves. The inoculated leaf sample was then kept at in 25°C and sealed it with parafilm so that humidity could be maintained. Then, after 1 day infection, phenotype was observed.

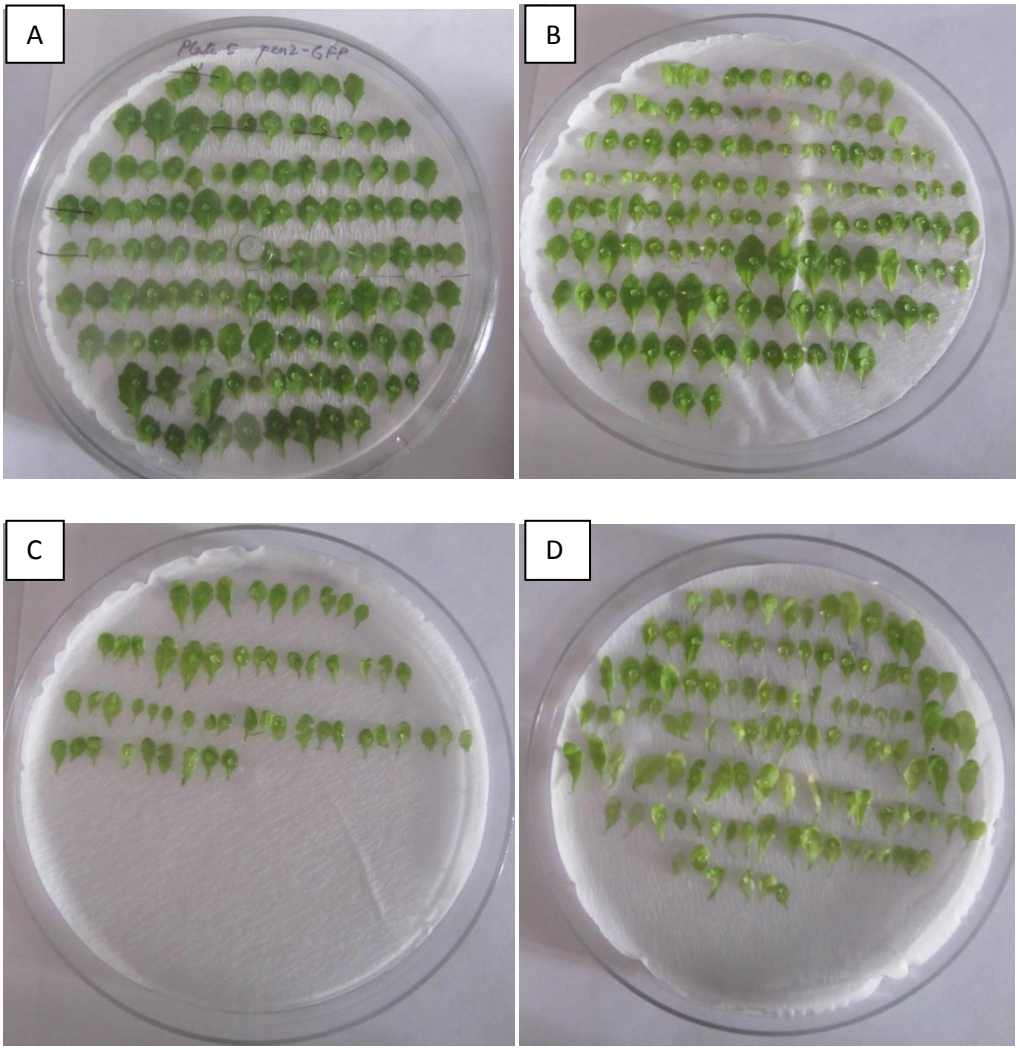


Figure-10 Phenotype of, leaf infection of *Arabidospsis* accessions by *Magnaporthe oryzae* spore

4.4 STAINING

For analyse the infection we take 1 day infected leaves for staining. Trypan blue stained the dead cell and aniline blue stained the callose deposition.

Fixing solution

Leaf samples were dipped in fixing samples (60% methanol, 30%chloroform and 10% acetic acid) and left for overnight.

4.4. a. trypan blue:

- Fixed sample were rehydrated through decreasing ethanol (100m80,70 and 50% ethanol)
- Samples then were stained in 0.05% trypan blue in distilled water overnight.
- De- staining was done in distilled water in next day.
- The leaves then were mounted in 30% glycerol on glass slides.

For visualization the stained cell, slides are observed under fluorescence and taken the images.

4.4. b. <u>Principle of Fluorescence microscopy</u>

The sample of interest is labelled with a fluorophore. Illuminated through the lens with the higher energy source (that may be a mercury or xenon lamp to produce ultraviolet light). Light comes into the microscope and hits a dichroic mirror. Dichroic mirror reflects the ultraviolet light up to the specimen. Ultraviolet light excites fluorescence within molecules in the specimen. The objective lens collects the fluorescent-wavelength light produced. This fluorescent light passes through the dichroic mirror (longer lower energy wavelength) and barrier filter (that eliminates wavelengths other than fluorescent) and making it to the eyepiece to form the image.

Figure-11 Diagram of fluorescence microscopy

4.4. c. Trypan-aniline blue combination

- Leaf samples were re hydrated through decreasing ethanol (100, 80, 70, and 50%).
- Samples were soaked in 0.05% trypan blue for overnight and then soaked in 0.05%aniline blue in 150mM KH_2PO_4, pH9.5 for 3-4 hr.
- The leaves then were distained in 150mM KH_2PO_4 and 2 to 3 times for 15 minutes and mounted on glass slides.

For visualization the stained cell, slides are observed under confocal microscope and taken the images.

4.4. d. <u>Principle of confocal microscope</u>

A laser is used to provide the excitation light (in order to get very high intensities). The laser light (blue) reflects off a dichroic mirror. From there, the laser hits two mirrors which are mounted on motors; these mirrors scan the laser across the sample. Dye in the sample fluoresces, and the emitted light (green) gets descanned by the same mirrors .The emitted light passes through the dichroic and is focused onto the pinhole. The light that passes through the pinhole is measured by a detector, i.e., a photomultiplier tube.

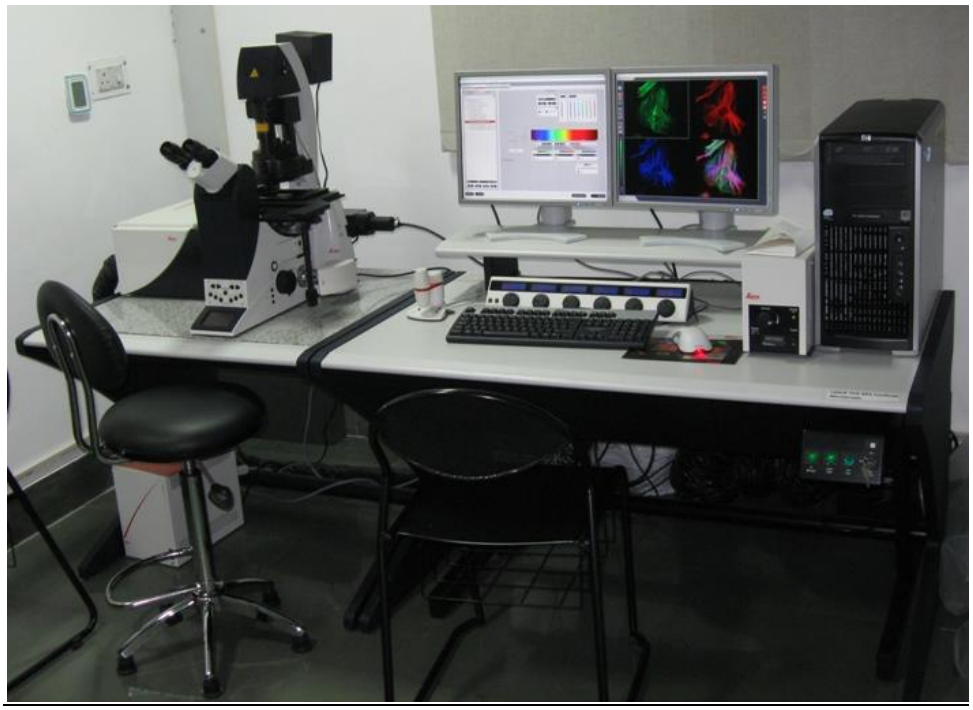


Figure-12 Diagram of confocal microscopy

4.5 DNA ISOLATION BY CTAB METHOD

4.5.1Materials required

4.5.1. a 2X CTAB buffer (for 10 mL)

NaCl–2.8mL from 5M NaCl stock

Tris HCl -1mL from 1M Tris stock

EDTA – 400µl from 0.5M EDTA stock

CTAB-0.2g

4.5.1. b TE buffer(for 10ml)

10mM Tris-100µl from 1M Tris stock

1mM EDTA- 20µl from 0.5M EDTA stock

For DNA isolation, 1 day infected plant was taken.

4.5.2 Procedure for DNA isolation

For DNA isolation, one-day infected plant was taken.

- About 100mg tissue was taken and mixed with CTAB buffer and grinding was done in mortal pastel.
- Incubation was done at 65°C about 30 minutes and cool at room temperature.
- About 700µl chloroform was added and vertexed at gently.
- Spinning was done at 12000g about 10 minutes in room temperature.
- Aqueous phase was taken.
- Isopropanol was added about 700µl and mixed well.
- Kept at room temperature and spinning at 12000g about 10 minute in room temperature.
- Supernatant was discarded.
- Ethanol (75%) was added about 500µl to pellet and spinning at 12000g for 3 minutes.

- Supernatant was discarded and pellet was air dry at room temperature about 2 minutes.
- About 20µl TE buffer was added to dried the pellet.

For visualization the DNA bands, DNA runs onto the agarose gel electrophoresis.

4.5.2.a Nano drop

To quantify the amount of DNA, check the tissue sample in Nano drop

Principle

DNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the DNA purity of DNA preparation. Pure DNA has an A260/A280 of 1.8.

Procedure

Firstly, set the DNA by taking buffer as blank. Then 1-2µl of DNA sample was taken and checked the concentration in µg/mL.

4.5.2.b. agarose gel electrophoresis

- DNA was checked through agarose gel electrophoresis. For preparing 1.2% gel, about 40 mL of 0.5X TBE buffer (5X TBE: 54g Tris , 21.5g boric acid, 20 ml 0.5M EDTA, pH 8) was mixed with 0.32gm of agarose and then the flask was covered with a film paper to avoid loss of liquid due to evaporation and boiled in microwave.
- Then it was kept for cooling and after that 1µl EtBr was added to it.
- It was poured onto the gel casting tray (BIO-RAD) and waited for a while until it was solidified.
- Then the DNA was loaded onto the well and it was run in TBE buffer with 8V/cm.
- After the gel was run (identified by the tracking dye, blue dye migrated upto 2/3rd of the gel length) then it check in the gel doc (BIO-RAD). DNA bands were documented in geldoc (BIO-RAD).

4.5.2.c. DNA Fragmentation

To check the integrity of the DNA, fragmentation was done

Procedure

First of all, 1.5% of agarose gel was prepared by adding 1µl EtBr.

Then, 5µg of DNA sample was taken and 3.2µl of gel loading dye was added in it.

Sample was loaded in the well along with ladder of 1500Kb.

The sample was run 80V/cm in TAE buffer up to two-third migration.

Then, DNA bands were documented in geldoc (BIO-RAD) and visualize that DNA is degraded or not.

4.6 RNA ISOLATION BY LiCl PRECIPITATION

4.6.1 Principle

Total RNA from the 300 mg leaf tissue of *Arabidopsis* was isolated by LiCl precipitation method .The plant tissue was grind in liquid nitrogen to fine powder and was mixed with buffer A/phenol in the ratio 1:3.

4.6.2 Materials Required

DEPC treated water (1000ml)

About 1000ml of distilled water was taken and 1ml of DEPC (diethylpyrocarbonate) was added in it. Stirring was done overnight by magnetic stirrer. Autoclaved it then repeated the above step once more and then it is ready for use.

Buffer A: Phenol (10ml)

Requirement:

8M LiCl :- 125µl

0.5M EDTA :- 200µl

20% SDS:- 500 µl

1M Tris pH9:- 1000 µl

DEPC treated water:- 8.175ml

Phenol:- 10ml

8M LiCl (125µl), 0.5M EDTA (200µl), 20% SDS (500 µl) and 1M Tris pH9(1000 µl) was added one by one in a falcon tube then maintained the volume by adding DEPC treated water.

After that equal volume of phenol was added in it. Before using it should be kept in the water bathat 80°C.

Phenol:- 10ml

For RNA isolation,1 day leaves sample was used.

4.6.3. Procedure for RNA isolation

For RNA isolation, one day infected leaves sample was used.

- About 300mg plant tissue was taken and grind in liquid nitrogen.
- Powdered tissue was mixed with about 1ml buffer A: Phenol which is highly heated at 80°C.
- Vertex was done about 5 minutes.
- About 500 µl chloroform was added and vertex about 5 minutes.
- Spinning was done at 12000g about 10 minutes.
- Aqueous phase was transferred to a fresh tube.
- About 500µl chloroform was added and spinned at 12000g about 5 minutes.
- Aqueous phase was transferred into a fresh tube.
- About 500µl 4M LiCl was added to the solution and vertex was done about 3 minutes.
- The tube was incubating overnight at -20°C.
- After overnight incubation spinning was done at 14000g about 20 minute in 4°C.
- Supernatant was discarded and pellet was resuspened in 300µl TE Buffer.
- Ethanol (100%) and NaOAc (3M) were added about 750µl and 30µl respectively.
- Incubation was done about 45 minutes at -20°C.
- After incubation spinning was done at 14000g about 20 minutes in 4°C.
- Supernatant was discarded and about 500µl ethanol (70%) was added to pellet.
- Spinning was done about 14000g about 10 minute in 4°C and supernatant was discarded.
- Pellet was resuspended with 20µl DEPC water and store at -80°C for future use.

In order to visualize the RNA band, agarose gel electrophoresis was done and documented in a gel doc (Bio-RAD).

4.6.3.a Nano drop

To quantify the amount of RNA, check the tissue sample in Nano drop

Principle

RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A260/A280 of 2.0.

Procedure

Firstly, set the RNA by taking buffer as blank. Then 1-2µl of RNA sample was taken and checked the concentration in µg/mL.

4.6.3.b. Agarose gel electrophoresis

- RNA was checked through agarose gel electrophoresis. For preparing a 1.2% gel, about 50 mL of 0.5X TBE buffer (5X TBE: 54g Tris , 21.5g boric acid, 20 ml 0.5M EDTA, pH 8) was mixed with 0.6gm of agarose and then the flask was covered with a film paper to avoid loss of liquid due to evaporation and boiled in microwave.
- Then it was kept for cooling and after that 2µl EtBr was added to it.
- It was poured onto the gel casting tray (BIO-RAD) and waited for a while until it was solidified.
- Then about 8-10 µl RNA was mixed with 2 µl bromophenol blue and was loaded onto the well and it was run in TBE buffer with 8V/cm.
- After the gel was run (identified by the tracking dye, blue dye migrated upto 2/3rd of the gel length) then it check in the gel doc (BIO-RAD). RNA bands were documented in geldoc (BIO-RAD).

4.6.4 DNaseI treatment:

- About 20µl RNA was taken.
- About 7µl DNase Buffer (10X) and 1µl DNaseI were added to RNA.
- Incubation was done at 37°C for 30 minutes and added DEPC water to maintain the final volume about 200µl.
- About 200µl phenol: chloroform (1:1) was added and vertexed.

- Spinning was done at 12000g for 10 minutes.
- Upper aqueous phase was transferred to fresh tubes.
- Chloroform was added about 200µl and vertexed.
- Spinning was done at 12000g for 5 minutes.
- Aqueous phase was transferred to fresh tubes.
- About 0.1 volume of NaOAc and 2.5 volume of ethanol (100%).
- Incubation was done for overnight at -20°C.
- Spinning was done at 12000g for 10 minutes and supernatant was discarded.

4.6.5 primers:

TABLE-3: List of primers used in this study of semi-quantitative RT-PCR

Oligo name	Len	MW	Tm	Mg/OD	OD	µg	nmol	2ndry	GC%	Ml for 100 µm	Seq
UBQ10F	22	6725	63.7	31.8	18.8	599.5	89.1	Very weak	54.4	891	GGCCTTGTATAATCCCTGATGA
UBQ10R	22	6868	60.5	27.5	17.3	476.5	69.3	none	36.3	693	AAAGAGATAACAGGACGGAAA
EF-1aF	22	6643	67.9	35.4	9.4	333.5	50.2	moderate	50	502	TGAGCACGCTCTTCTTGCTTTC
EF-1aR	22	6772	67.8	32.6	14.8	482.8	71.2	weak	50	712	GGTGGTGGCATCCATCTTGTTA
FRK1F	19	5871	59.9	29.8	8.7	259.5	44.2	none	52.6	442	GCCAACGGAGACATTAGAG
FRK1R	20	6006	59.6	32.0	12.2	391.4	65.1	None	50	651	CCATAACGACCTGACTCATC
NHL10F	20	5997	63.6	32.8	21.8	716.7	119.5	None	50	1195	TTCCTGTCCGTAACCCAAAC
NHL10R	20	6118	63.7	32.1	17.5	562.3	91.9	Weak	60	919	CCCTCGTAGTAGGCATGAGC
CYP81F2F	22	6835	63.0	28.7	14.2	407.9	59.6	None	40.9	596	AAATGGAGAGAGCAACACAATG
CYP81F2R	20	6012	63.4	32.3	14.7	475.1	79.0	Very weak	45	790	ATCGCCCATTCCAATGTTAC
PR1F	22	6825	67.9	31.2	14.2	443.1	64.9	None	54.5	649	AAAACTTAGCCTGGGGTAGCGG
PR1R	24	7199	66.2	33.6	15.3	514.4	71.4	None	45.8	714	CCACCATTGTTACACCTCACTTTG
PDF1.2aF	22	6858	66.7	29.8	11.7	348.8	50.8	Very weak	50	508	AGAAGTTGTGCGAGAAGCCAAG
PDF1.2aR	23	7160	66.8	31.5	13.3	419.8	58.6	Very weak	52.1	586	GTGTGCTGGGAAGACATAGTTGC

4.6.6 cDNA preparation

First of all, 18µl of template RNA was mixed in 1µl primer

Then, RNA sample was incubated on 70°C for 2 minutes

After that, sample was placed in Ice for 2 minutes

10µL of buffer plus 5µl of dNTP was added in RNA sample

Then 1µl of Reverse Transcriptase was added in it

Then, 15µl of DEPC treated water was added in the RNA sample and mixed it.

Then the sample was run on PCR.

Table:**4** cDNA Protocol

Reaction volume 50µl	Time
25°C	10 min
37°C	1: 30:20
75°C	15 min
10°C	∞

After this we got the cDNA, then we run the sample in normal PCR

Table:5: PCR programme in thermal cycler (Bio-RAD)

PCR (Vol- 10µl)	Time	
94°C	3 min	
94°C	30 sec	
55°C	20 sec	34 cycle
72°C	45 sec	
72°C	10 min	
4°C	∞	

After completing the PCR, cast 2% agarose gel in 1X TAE buffer, load the sample and run. The gel should run up to two-third. Then it was observed the band in UV trans-illuminator.

5. RESULT AND DISCUSSION:

A) Phenotypic result shows after infection on leaves surface.

TABLE-6 (COL0 PLANT)

RRR	RRR	RRR	RRR
RRR	SSS	RRR	RRR
RRR	RRS	RRS	RRS
RSS	RRR	RRS	RRR
RRS	RRR	RRS	RSS
RRR	RRR	RRR	RRR
RRS	RRR	RRR	RRR
RRR	SSS	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRS	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR			

TABLE-7 (PEN2-3 PLANT)

RRR	RRR	RRR	SSS
RRR	RRR	RSS	RRS
RSS	RRS	RRR	RSS
SSS	RRR	SSS	RSS
RSS	RRR	RRR	RSS
RRR	RRS	RRR	RRR
RRS	RSS	RRR	RRR
RRR	RRR	RR_	RRR
RRR	RRS	RRR	RRR
RRS	RRS	RRS	RRS

RRR	RRR	RRS	RRS
RRR			

TABLE -8 (PEN2 GFP)

RRR	RRR	RRR	RRR
_RR	RRR	RRR	RRR
RRR	RRR	RRR	R_ _
RR_	RRR	RRR	RRR
RRR	RRS	RRR	RRR
RRR	RRR	R_ _	RRR
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RR_
RRR	RRR	RRR	RRR
RRR	RRR	RRR	RRR
RRR			

TABLE-9 (PEN 2-3,PEN3-1 PLANT)

RRR	RRR	RRR	_ _R
_RR	RRS	RSS	RSS
RSS	SSS	RSS	RSS
RSS	RRS	RRS	RRS
_SS	R_ _	RRS	

TABLE-10 (PEN 3-1 , PEN 1-1 PLANT)

RRR	RRR	RRR	RRR
RRR	RR_	RRR	RRS
RRR	RRR	RR_	RSS
RRS	_ _ _	_ _ _	RRS
RRS	_ _ _	RRS	R_ _
RRS	RRS	RR_	RS_

RRR	_ _R	RRS	RRS
RRS	RRS	R_ _	RSS
RRS	RRS	_ _ _	R_ _
R_S	RRS		

Percentage (%) of resistant and susceptible

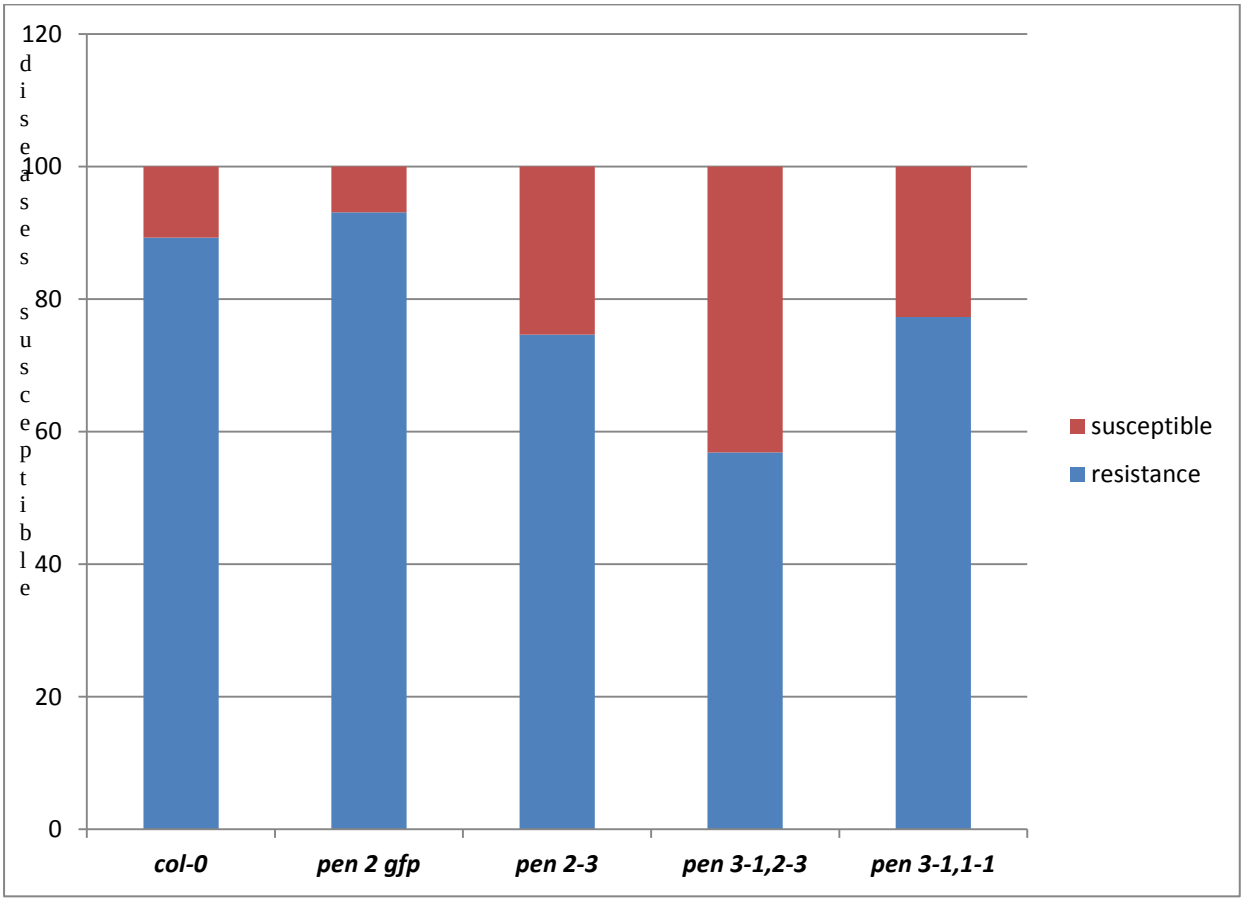


Figure-13- Graph percentage (%) of resistant and susceptible

Here the comparison between *col-0, pen 2 gfp, pen 2-3,pen 3-1,2-3, pen 3-1,1-1* were performed. In the table 4-8, "R" represents the resistant and "S" represents the susceptible. According to resistance and susceptibility, *pen 3-1, 2-3* are having highest susceptibility and *pen 2 gfp* having low susceptibility. In *pen 2 gfp*, the percentage of resistance is higher than double mutant.

B) <u>Fluroscence microscopy (Trypan blue staining)</u>

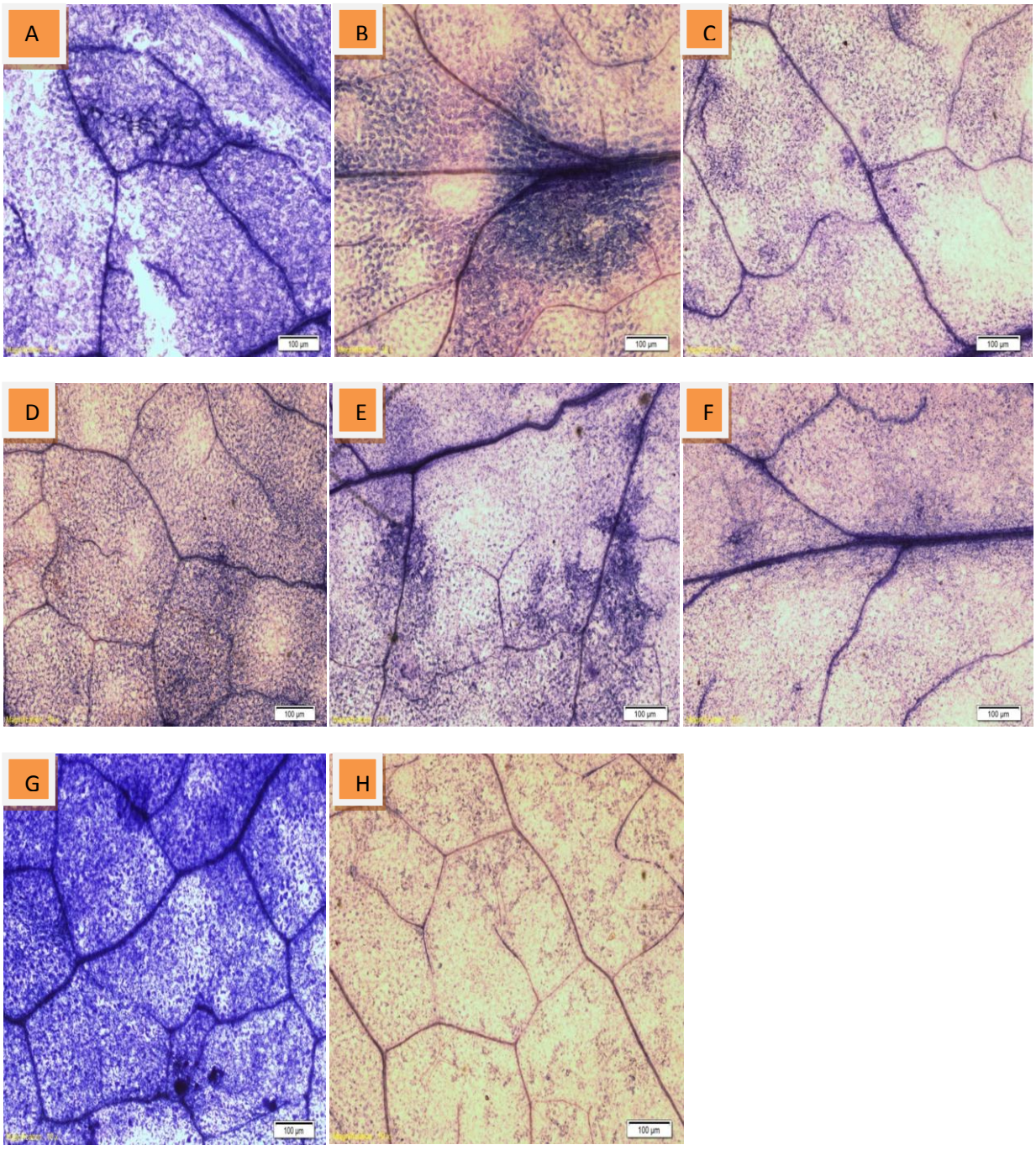


Figure-14 picture of trypan blue staining

A)Pen 2 GFP [water treatment] B) Pen 2 GFP [1 day infection] C)Pen 2-3 [water treatment] D) Pen 2-3 [1 day infection] E) Pen 3-1,1-1 [water treatment] F) Pen 3-1,1-1 [1 day infection] G) Pen 3-1,2-3 [water treatment] H) Pen 2 GFP [1 day infection]

One day infection leaves were seen under the fluorescence microscope to observe the rate of infection. But in fluorescence microscope, infections are not clearly visible. So confocal microscopy was done.

C) Confocal microscopy (Trypan and aniline blue combination; dual staining)

pen 2-3-

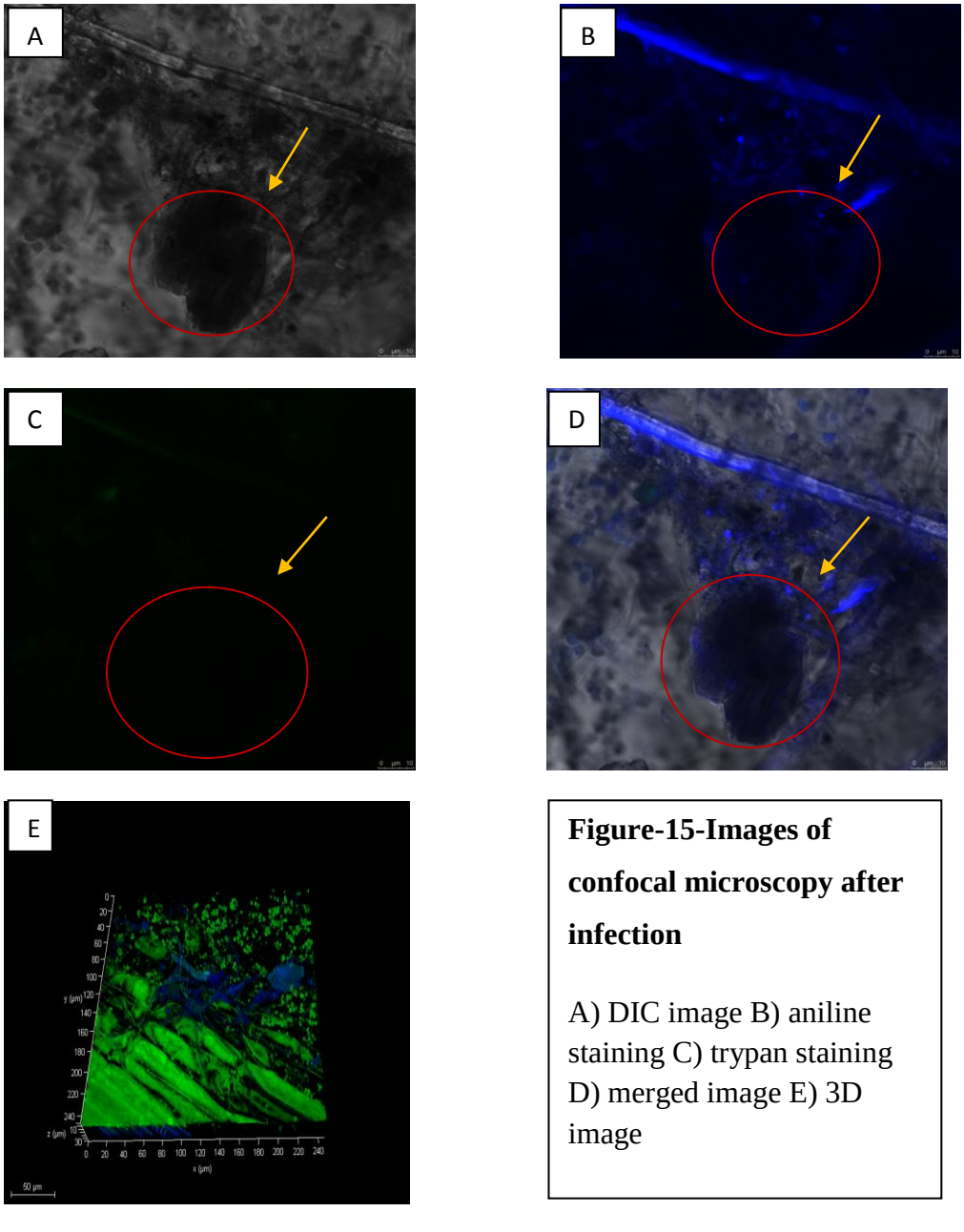


Figure-15-Images of confocal microscopy after infection

A) DIC image B) aniline staining C) trypan staining D) merged image E) 3D image

The red circles are shown the dead cell after the infection. 3D images are showing the depth of the infection.

pen 3-1, 1-1

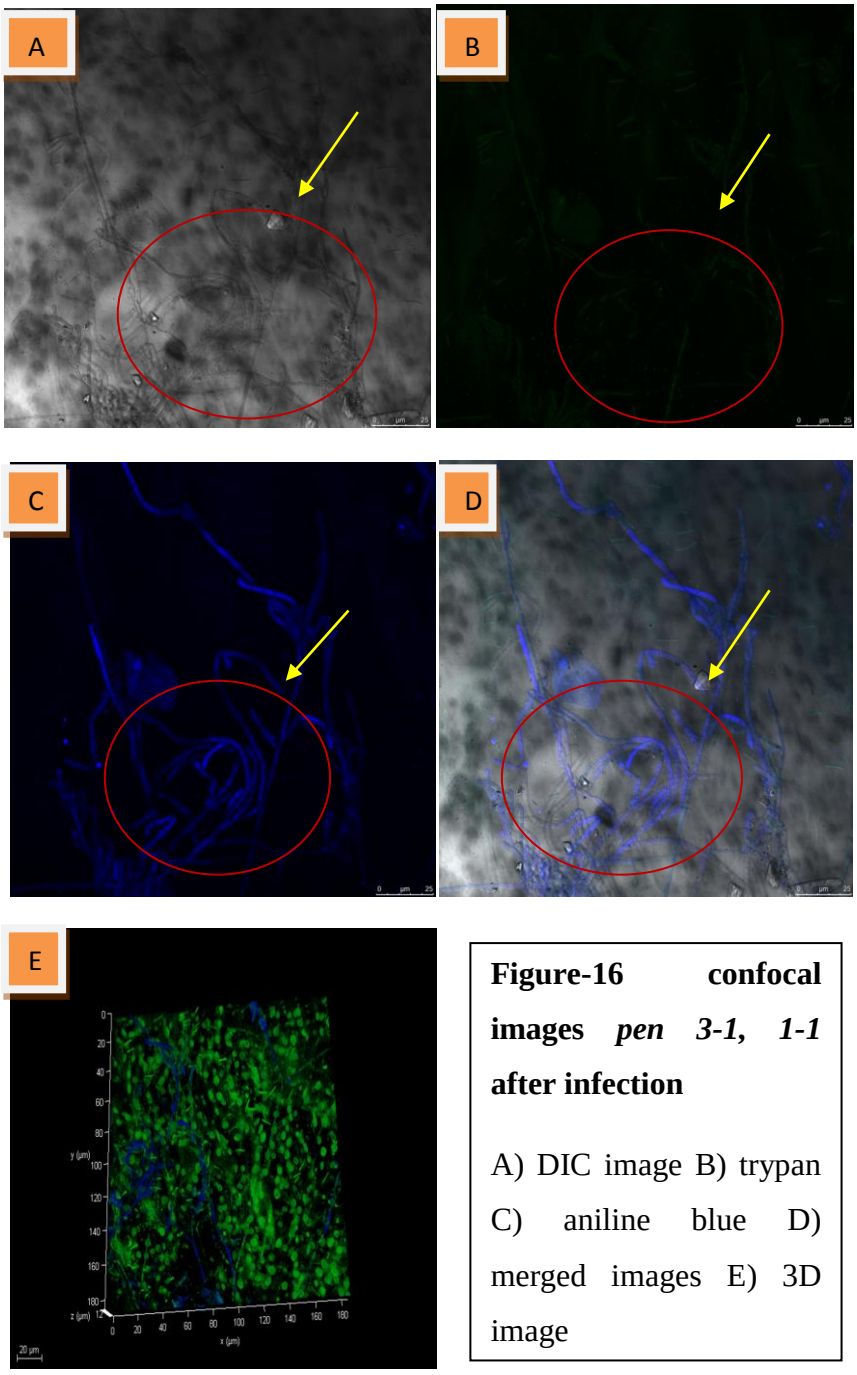


Figure-16 confocal images *pen 3-1, 1-1* after infection

A) DIC image B) trypan C) aniline blue D) merged images E) 3D image

The red circles are shown the infection (hyphal growth). 3D image shown the depth of the infection. The infections are shown that more than single mutant pen 2-3.

pen 3-1,2-3

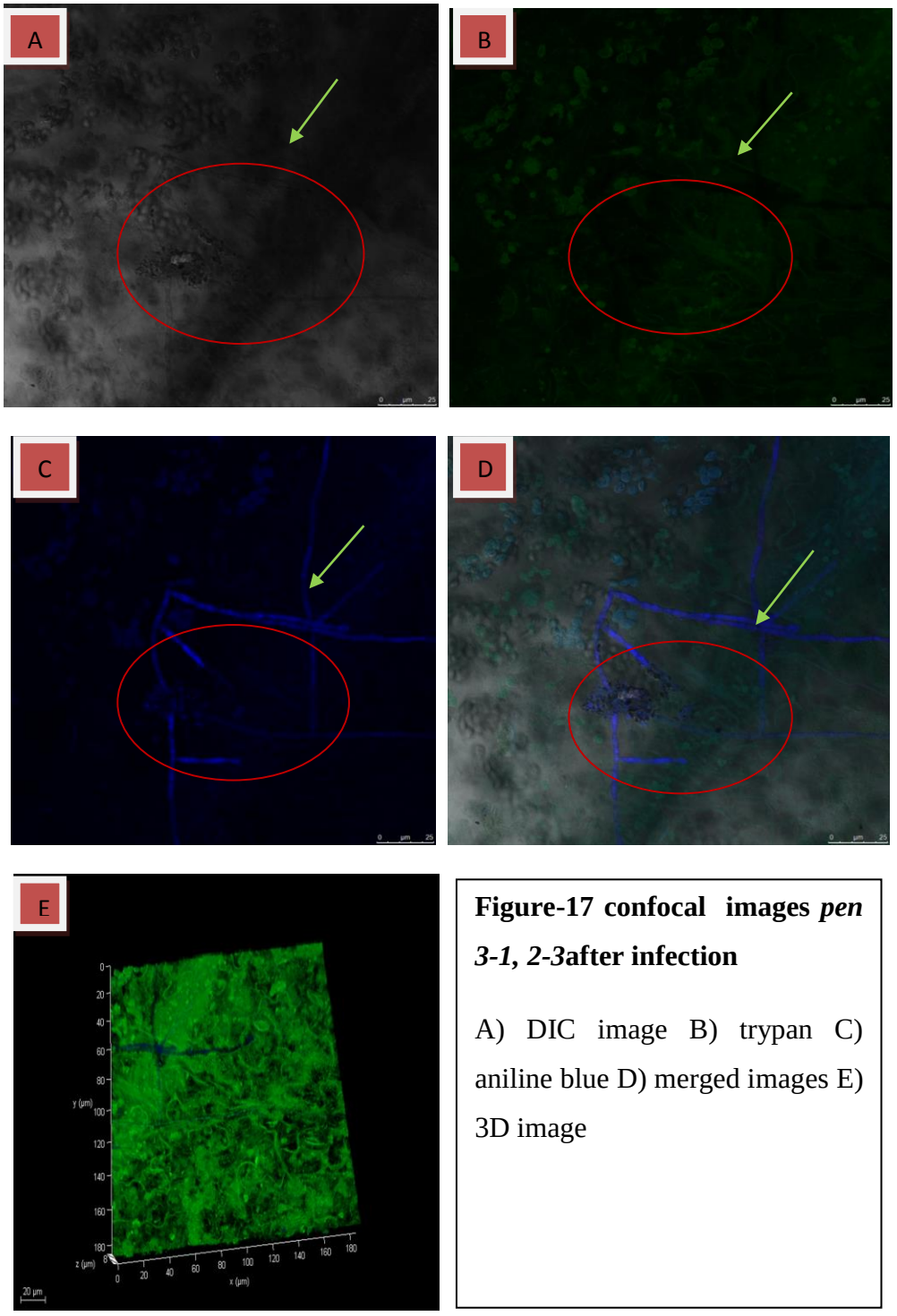
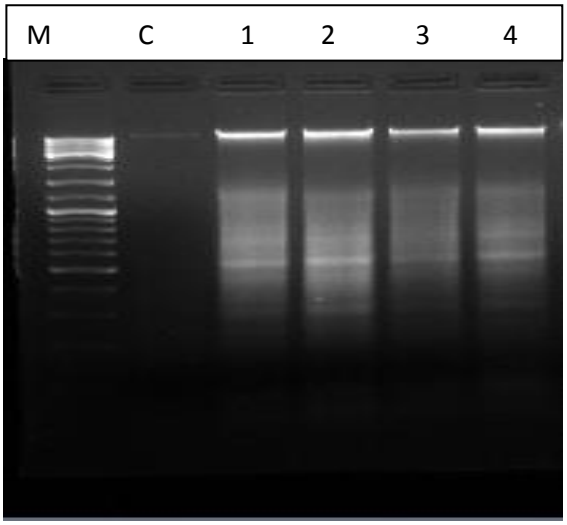


Figure-17 confocal images *pen 3-1, 2-3*after infection

A) DIC image B) trypan C) aniline blue D) merged images E) 3D image

The area occupied by red circle which is infected by *M. oryzae.* In pen 3-1, 2-3 more infection is occurred than single mutant.

D) To check the DNA integrity after infection by *M. oryzae*.



M-ladder

C-control

1- pen 2 gfp

2-pen 2-3

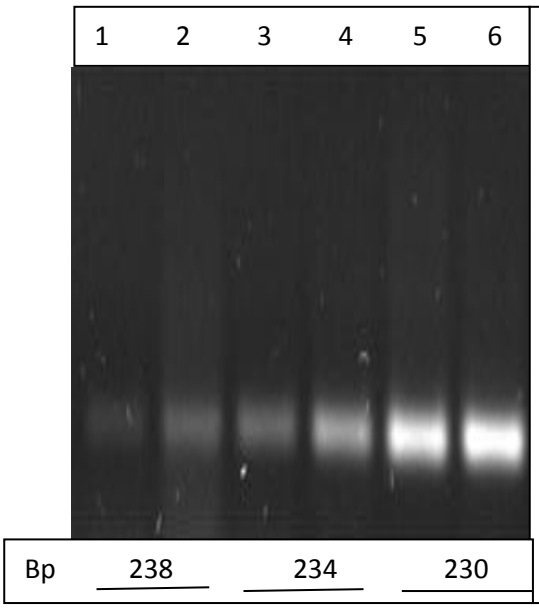
3-pen 3-1, 1-1,

4-pen 3-1, 2-3

Figure-18 Bands patterns are shown

The DNA in the agarose gel shown as many bands. This indicated that DNA is degraded by *M.oryzae* into many bands. But in control plant there is no infection was occured so that there is no bands was shown.

E) To check the expression pattern of defense genes during infection in *pen2-3*, RT-PCR for *PR1*, *PDF1.2* and *UBQ* (internal control) was done



Samples and treatments

1 – *PR1* water

2 – *PR1* infection

3 – *PDF1.2* water

4 - *PDF1.2* infection

5 - UBQ water

6 – *UBQ* infection

Figure-19- expression pattern of defence gene

From 1st and 2nd lane shows that *PR1* gene is more expressed in infected leaves, 3rd and 4th lane indicated that *PDF 1.2* gene expressed more in infected leave (4th lane). Lastly 5th and 6th lane indicated the role of ubiquitin which is act as internal control of primer.

6. CONCLUSION

The conclusion from the overall experiment is that it shows the infection pattern in mutant (*pen 2-3, pen 3-1, 2-3, pen 3-1,1-1*) *Arabidopsis* by *M. oryzae.* In double mutants, infection is more than single gene mutant. Mutants act as susceptible to *M. oryzae* with their compromising NHR against the pathogen. Defense genes are (*PR1, PDF*) activated during the infection and they highly expressed suggesting that the SA and JA pathways are activated with JA signaling pathway the most as we see *PDF1.2* marker gene expression to highly induce upon infection at 1 dpi.

7. FUTURE WORK PLAN

The genes which provide the non-host resistance to *Arabidopsis thaliana* were identified. If the resistance gene can be transferred to the rice, then the rice germplasm may show resistance against the rice blast pathogen caused by *M. oryzae.* The percentage of rice blast disease will be decreases and it will be helpful for the farmer and more healthy cultivation will be done. The new germplasm generated will provide broad spectrum, durable resistance and at the same time the germplasm will be eco-friendly.

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